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Comparison of intestinal permeability, morphology, and ileal microbial communities of commercial hens housed in conventional cages and cage-free housing systems

Maddison L. Wiersema
Iowa State University, wiersema@iastate.edu

Lucas Koester
Iowa State University, lkoest12@iastate.edu

Stephan Schmitz-Esser
Iowa State University, sse@iastate.edu

Dawn A. Koltes
Iowa State University, delkins@iastate.edu

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Comparison of intestinal permeability, morphology, and ileal microbial communities of commercial hens housed in conventional cages and cage-free housing systems

Abstract

The gastrointestinal health of poultry can be impacted by a variety of factors including their environment. As egg production moves from conventional cage housing (CC) towards cage-free housing (CF), it is important to understand this impact on intestinal health. This study was conducted to determine if housing type impacted intestinal permeability, morphology, and microbial communities in commercial hens across housing systems. Hens were randomly selected from 2 rooms of CC (n = 25) and CF (n = 25) at a commercial facility. Birds were given fluorescein isothiocyanate dextran (FITC-D) by oral gavage to measure intestinal permeability. Jejunal and ileal samples were collected to evaluate villus height, crypt depth and their ratio. Ileal contents were collected for bacterial DNA isolation and 16S rRNA gene sequencing. Serum FITC-D was similar between housing type (P = 0.709). Hens housed in the CF had increased jejunal villus height and crypt depth compared to hens from the CC (P < 0.002). Hens from the CC tended to have a greater villus height to crypt depth ratio in both the jejunum and ileum compared to the CF (P = 0.064; P = 0.091, respectively). Microbial community diversity measurements favored hens housed in the CC as ileal contents tended to have increased species richness (P = 0.059), had greater alpha diversity (P = 0.044), and had an increased number of over represented OTUs (46/64), including *Romboutsia sp.* (30.80%), *Lactobacillus kitasatonis* (17.16%), and *Lactobacillus aviarius* (11.15%). Correlations between microbial communities with intestinal traits identified significant association with the greatest number of correlations with FITC-D and ileal morphology. Many of these correlations identified microbial communities associated with expected traits; thus, providing limited functional data to microbial communities with limited information. The greater number of correlations of ileal morphology with ileal microbial communities suggesting local microbial communities contribute to the intestinal environment distant. In this limited study, several parameters favored hens from CC suggesting an advantage of this system for intestinal health. However, the lower intestinal health parameters observed in CF were not at levels to indicate detrimental effects.

Keywords

fluorescein isothiocyanate dextran, jejunum, ileum, Lactobacillus, villus height to crypt depth ratio

Disciplines

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RUNNING TITLE: HOUSING TYPE AND HEN INTESTINAL PARAMETERS

Comparison of intestinal permeability, morphology, and ileal microbial communities of commercial hens housed in conventional cages and cage-free housing systems

Maddison L. Wiersema[*^], Lucas Koester[‡^], Stephan Schmitz-Esser[*], and Dawn A. Koltes[*†]

*Department of Animal Science, Iowa State University, Ames, Iowa 50011

^Shared first authorship

[‡]Vet Microbiology & Preventive Medicine and Interdepartmental Microbiology Graduate Program, Iowa State University, Ames, IA

†Corresponding Author:

Dawn A. Koltes

1029 N. University Blvd

3101 NSRIC

Ames, Iowa 50011

Phone: 515-294-5694

delkins@iastate.edu

Section: Physiology and Reproduction

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ABSTRACT

The gastrointestinal health of poultry can be impacted by a variety of factors including their environment. As egg production moves from conventional cage housing (CC) towards cage-free housing (CF), it is important to understand this impact on intestinal health. This study was conducted to determine if housing type impacted intestinal permeability, morphology, and microbial communities in commercial hens across housing systems. Hens were randomly selected from 2 rooms of CC (n = 25) and CF (n = 25) at a commercial facility. Birds were given fluorescein isothiocyanate dextran (FITC-D) by oral gavage to measure intestinal permeability. Jejunal and ileal samples were collected to evaluate villus height, crypt depth and their ratio. Ileal contents were collected for bacterial DNA isolation and 16S rRNA gene sequencing. Serum FITC-D was similar between housing type ($P = 0.709$). Hens housed in the CF had increased jejunal villus height and crypt depth compared to hens from the CC ($P < 0.002$). Hens from the CC tended to have a greater villus height to crypt depth ratio in both the jejunum and ileum compared to the CF ($P = 0.064$; $P = 0.091$, respectively). Microbial community diversity measurements favored hens housed in the CC as ileal contents tended to have increased species richness ($P = 0.059$), had greater alpha diversity ($P = 0.044$), and had an increased number of over represented OTUs (46/64), including *Romboutsia sp.* (30.80%), *Lactobacillus kitasatonis* (17.16%), and *Lactobacillus aviarius* (11.15%). Correlations between microbial communities with intestinal traits identified significant association with the greatest number of correlations with FITC-D and ileal morphology. Many of these correlations identified microbial communities associated with expected traits; thus, providing limited functional data to microbial communities with limited information. The greater number of correlations of ileal morphology with ileal microbial communities suggesting local microbial communities contribute to the intestinal

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environment distant. In this limited study, several parameters favored hens from CC suggesting an advantage of this system for intestinal health. However, the lower intestinal health parameters observed in CF were not at levels to indicate detrimental effects.

Key words: fluorescein isothiocyanate dextran, jejunum, ileum, Lactobacillus, villus height to crypt depth ratio

INTRODUCTION

An increasing number of companies are pledging to no longer sell, serve, or utilize eggs from hens housed in conventional cages (CC). In addition to changes in general operations as farmers transition to cage-free systems (CF; Ward, 2014; Xin et al., 2012), much remains unclear regarding how these systems alter the physiology of the hens living in them in response to increased mobility and exposure to excreta. Understanding these differences will be critical for maximizing efficiencies and animal welfare.

The shift from CC increases the overall area a hen can move and is required to move as nest boxes, water lines, and feed lines are at increased distances compared to the CC system. Therefore, it was not surprising that numerical increases in energy requirements were observed in commercial hens raised in CF system compared to CC (Karcher et al., 2015). While this difference could be due to the increase in energy exerted in the form of movement, it is unclear if there are changes in intestinal morphology or permeability that can alter digestibility of consumed nutrients.

In addition to increasing the distance hens need to move for daily activities, the additional interaction with the environment including excreta may lead to chronic inflammation, dysbiosis or enteric disease. While no differences in eggshell contamination with *Salmonella* or *Campylobacter* were observed between housing systems, the observed increase in environmental

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contamination in the CF systems would suggest differences in how the hens interact with the environment (Jones et al., 2015). Therefore, these reduced hygienic conditions may put hens at an increased risk for colonization of microbial communities that compete for nutrients, secrete metabolites that suppress production, reduce nutrient digestion and absorption, increase subclinical infections and/or allow for colonization of detrimental bacteria**.** A recent publication by van Goor, et al. (2020), characterized microbial communities in the ceca of CF and CC hens. While they did not directly compare microbial communities between housing systems, microbial diversity of the ceca remained similar across stage of lay with CC, but not with CF; suggesting the environment may alter stability of the microbiome which in turn may alter nutrient digestion and absorption (van Goor et al., 2020). To understand the effects of hen housing system on intestinal health which will contribute to nutrient digestion and absorption, it is critical to determine changes in bacterial communities and gastrointestinal health. Therefore, this study was designed to characterize bacterial communities, whole intestine permeability, and intestinal morphology between CF and CC systems and to determine associations between resident microbes and intestinal parameters.

MATERIALS AND METHODS

Animals

All procedures involving animals were approved by Iowa State University's Institute of Animal Care and Usage Committee (IACUC number 18-231).

Twenty-five hens were randomly chosen and weighed from 2 different rooms of either a CF (n=50) or CC (n=50) housing system at a single commercial layer facility in Iowa. Hens within each room were the same age; however, the ages of the hens between rooms ranged from 26 to 70, and it is expected hens fed on performance/age appropriate diets. As the focus of this

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trial was to characterized parameters between commercial CF and CC systems, we chose to treat differences of management such as dietary formulation as a factor that is confounded with CF and CC systems.

Intestinal Permeability

Hens selected from CF and CC systems were orally inoculated with Fluorescein isothiocyanate–dextran average molecular weight 3000-5000 (FITC-D; Sigma Aldrich, FD4) at a rate of 16.64 mg/ml according to a previously described protocol by Baxter et al., (2017). Two hens per room were not inoculated and were used for control serum. One hour after hens were inoculated with FITC-D, hens were euthanized via cervical dislocation. Blood samples were collected from the femoral artery into serum blood collection tubes (BD367815; Fisher Scientific) and transported back to Iowa State University on ice for serum separation (10,000 x g for 15 minutes). Once the serum was separated, it was aliquoted and stored at -80°C in amber tubes to prevent break down of the fluorescence until analysis. All samples from hens given FITC-D were diluted at a ratio of 1:5 in saline. Using serum from control hens, a standard curve was generated for FITC-D. Diluted samples were plated in triplicate. Fluorescence was measured using a BioTek Cytation fluorescence spectrophotometer (BioTek US, Winooski, VT) with excitation and emission wavelengths of 485 and 528 nm, respectively. For data analysis, triplicates were averaged for each hen.

Jejunum and Ileum Morphometric Analysis

After euthanasia, a 2 cm section of the jejunum at Meckel's Diverticulum and of the ileum 5 cm proximal of the ileocecal junction was quickly excised, flushed with phosphate buffered saline, and placed in 10% formalin buffered saline. Formalin fixed samples were sectioned, embedded, and stained with hematoxylin and eosin stain by the Iowa State University

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Veterinary Histopathology Lab. Additionally, the ileal samples were stained with Alcian blue to determine goblet cell number. Images used for morphometric measurements (villus height and crypt depth) and cells counts were captured using an Olympus BX63 microscope and camera. Ten morphometric measurements per parameter were determined using the ImageJ software (Schindelin et al., 2012; Schnieder et al., 2012). Goblet cells were counted for the entire area of the image using color and shape filters in Image J. Data is expressed in counts per mm^2. Three images per bird were used for analysis.

Characterization of Bacterial Communities and Sequence Analysis

Ileal luminal contents were aseptically removed from a 5 cm section adjacent and proximal to the section collected for morphometric analysis. Samples were transported on dry ice back to Iowa State University and stored at -80°C until DNA isolation. DNA from these ileal samples was extracted using the Qiagen Powerlyzer soil kit following the manufacturer's recommendations. After confirming DNA concentrations using a nanodrop (ND 2000; Fisher Scientific), 90 samples were found to contain DNA and were used to amplify bacterial and archaeal 16S rRNA genes. Samples were sequenced using 250 bp paired-end reads for each sample of the V4 region of the 16S rRNA gene (515F, 806R; Caporaso et al., 2011; Caporaso et al., 2012) at the Iowa State University DNA Facility using Illumina MiSeq sequencing technology.

Sequence analysis was done with mothur V1.40.4 following the mothur MiSeq Standard Operating Procedure (Kozich et al., 2013). Barcode sequences, primers and low-quality sequences were trimmed using a minimum average quality score of 35, with a sliding window size of 50 bp. Chimeric sequences were removed with the "Chimera.uchime" command. For alignment and taxonomic classification of operational taxonomic units (OTUs), the SILVA SSU

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NR reference database (V132) provided by the mothur website was used. Sequences were clustered into OTUs with a cutoff of 99% 16S rRNA gene similarity (=0.01 distance).

To compare alpha diversity between experimental groups, reads were randomly subsampled to accommodate the sample with the lowest number of reads across data sets (20,000 sequences). Measurements of Chao species richness, Shannon diversity, and Simpson evenness were taken to compare community structures between experimental groups.

Average Bray-Curtis dissimilarity measures for each treatment group were compared using the analysis of similarity (ANOSIM) package provided by mothur (Clarke, 1993; Schloss et al., 2009). Bray-Curtis was selected as the dissimilarity coefficient because of its ability to compare closely related samples.

All plotting was completed using ggplot2, v2_3.1.1 graphing package (Wickham, 2016; R Core Team, 2019) in R 3.6.0. Overall variation in bacterial communities were visualized using principle coordinate analysis (PCoA). This information was generated with the Phyloseq (v1.28.0 (McMurdie and Holmes, 2013)) and Vegan (v2.5-5, (Oksanen et al., 2019)) packages using the shared and taxonomy file generated in mothur. Sequences were randomly subsampled to 20,000 sequences and Bray-Curtis dissimilarity measures were used to generate distances between samples for the PCoA plot.

Statistical Analysis

Differences for intestinal permeability, morphology, microbial community parameters, and individual OTUs were determined across housing type using PROC Glimmix in SAS (SAS Institute Inc., 2011) with housing type fit as a fixed effect and room fit as a random effect. Significance was set at a $P < 0.05$. To determine if specific bacterial OTU abundances were significantly different across housing type, data were normalized using the trimmed mean of the

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M-value (TMM; Robinson and Oshlack, 2010) for the top 200 OTUs and had at least 2 reads in 45 of the 90 samples. Data were then analyzed using PROC Glimmix in SAS for each OTUs following a negative binomial distribution and using housing type as a fixed effect (SAS Institute Inc., 2011). q-values were used as a means to control for false discovery rate using the q-value package in R (Storey et al., 2004). For OTUs, significance was set at a $P < 0.05$ and $q < 0.05$. To determine potential beneficial or detrimental bacterial communities, correlations were determined between bacterial communities and intestinal leakage or morphometric measurements using PROC CORR within housing type. Significance was set at a $r^2 > |0.35|$.

Data availability

The 16S rRNA gene sequences have been submitted to the NCBI Sequence Read Archive SRA and are available under the BioProject ID PRJNA647366.

RESULTS AND DISCUSSION

Animal Parameters

All hens used for this study were apparently healthy at the time of selection. The average body weight of hens included in this study was 1.4 kg (1.42 $\pm$ *0.06* kg for CF; 1.41 $\pm$ *0.06* kg for CC $P = 0.978$).

Intestinal Parameters

Macromolecular flux of FITC-D, a non-digestible sugar, from the lumen of the intestine into circulation, was not altered by housing type ($P = 0.348$; See Table 1). Due to the low levels of FITC-D in the serum, a large number of samples were not above the lowest standard. To ensure this was not bias to a single treatment we also ran the samples based on fluorescence. Again, no difference was observed by housing type ($P = 0.709$; See Table 1). The lack of difference and low detection was expected as these birds were presumably healthy and on feed,

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two factors that are experimentally used to induce elevated intestinal permeability of FITC-D (Baxter et al., 2017; Vicuña et al., 2015). However, we observed high hen-to-hen variation across housing type indicating that the individual hen interaction with the environment had more effects on intestinal permeability than housing system. While average hen weights were not significantly different across housing treatment, individual hen weights did vary; however, the inclusion of body weight as a covariate into the statistical model did not alter the results observed when weight was not included ($P = 0.656$).

Given the jejunum is the area of highest digestion and absorption, significant changes in this region could indicate changes in digestion and absorption across housing types. In this study, jejunal villus height and crypt depth were increased in hens from the CF system compared to hens from the CC system ($P < 0.002$; See Table 1). Villus height to crypt depth ratio tended to be greater in hens from the CC system compared to the CF system ($P = 0.064$; Table 1). Additionally, to observe changes where microbial populations increase and assist in the last of the small intestine digestion and absorption, ileal morphometric parameters were measured. Ileal villus height and crypt depth were not different between hens from the different housing systems. However, the villus height to crypt depth ratio tended to be greater in hens from CC systems ($P = 0.091$; Table 1) and number of goblet cells were increased in hens from CC ($P < 0.001$; Table 1).

Intestinal morphology is used as an indicator of intestinal health as values are often indicative of digestive and absorptive capacity. For both jejunum and ileum, villus height and crypt depth ratio were similar to previously reported length (Applegate et al., 2009; Deng et al., 2012; Pereira et al., 2019) indicating that intestinal absorption capacity was within normal ranges and not indicative of diseased states. In our study, hens from the CF system had increased villus height in the jejunum, an area of high nutrition absorption, as well as increased crypt depth

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suggesting these hens may have higher intestinal absorption while continuing to proliferate new cells for the intestinal lining. This continued production of cells by the intestine is energetically unfavorable. Therefore, the ratio of the villus height to crypt depth is often used as a single measure of intestinal health. Surprisingly, the villus height to crypt depth ratio tended to be increased in the jejunum and ileum of CC hens suggesting the intestine of these hens is more favorable ($P < 0.092$; Table 1). Extreme changes in villus height and crypt depth are observed during times of disease or toxin challenge with villus height decreasing as dying cells are removed and crypt depth increasing to support new cell growth (Yason et al., 1987). Extrapolation of these measures are often applied in non-disease challenges when changes are more subtle, as is the case in this study, and should be done cautiously.

Ileal Microbial Communities

Taxonomic assignment. 250 bp paired-end MiSeq sequencing of the 90 samples resulted in 8,570,879 raw sequences. After removing low-quality sequences, 6,474,777 sequences remained, which were clustered into 46,018 OTUs. Both the SILVA SSU NR reference database (V132) provided by the mothur website and NCBI Blast on representative sequences were used to assign OTUs a taxonomic classification and are provided in all tables where OTU data is present.

Alpha diversity measurements. This is the first study to examine the changes in ileal microbial communities in laying hens across different housing environments. With the exception of evenness (Simpson index; $P = 0.387$), average ileal species richness (Chao index) tended to be higher ($P = 0.059$) and overall alpha diversity (Shannon index) was higher for hens housed in CC systems ($P = 0.044$; Figure 1). Results from this study suggest the species richness and alpha diversity of the microbial communities are more favorable in CC systems; which may

provide greater plasticity of bacterial communities. However, the spread or evenness of microbial communities was similar. This evenness of microbial communities was, also, observed in cecal contents from hens housed in CF and CC systems (Hubert et al., 2019) potentially suggesting some structure or order to how microbial communities are allowed to flourish in the chicken intestine. Interestingly, Hubert et al. (2019) observed greater alpha diversity in cecal content of hens from CF systems; while van Goor et al., (2020) observed greater alpha diversity in cecal contents of hens from conventional cage systems. While these communities were collected from different regions of the digestive system compared to this study, it should, also, be pointed out that hens housed in the CF environment in the Hubert et al. (2019) study had access to outdoor spaces while hens in this study did not. While outdoor access was not mentioned in van Goor et al., (2020), the differences in alpha diversity measurements for these microbial communities may not only be a result of intestinal segment, but access to outdoor microbes.

Beta diversity measurements. Whole community Beta diversity comparisons of CF and CC microbial community samples were made using Analysis of similarity (ANOSIM) and Analysis of molecular variance (AMOVA) comparing average Bray-Curtis distances per group and found significant differences in microbial communities between housing types (ANOSIM; P = 0.0003 and AMOVA; P = 0.004; Figure 2). However, PCoA plots revealed no clear clustering of the microbial communities based on housing type.

Ileal microbial communities. At the phylum level, 21 phyla were identified from samples between both housing types (Supplemental Figure 1). The majority of phyla were *Firmicutes* (91.5%), *Proteobacteria* (1.83%), *Fusobacteria* (0.85%), and *Actinobacteria* (0.63%). The major genera found in both housing types included mainly *Lactobacillus* (45.0%), *Romboutsia* (34.8%), *Tyzzerella* (3.74%), *Candidatus Arthromitus* (3.47%), *Gallibacterium*

(1.76%), and *Turicibacter* (1.32%; Supplemental Figure 2). The percentage of phyla are similar to previously published ileal microbiome communities in laying hens (Ngunjiri et al., 2019; Wang et al., 2019).

In hens from the conventional cage system, *Romboutsia* was the most abundant genus (30.80%), followed by two *Lactobacillus* phylotypes: *Lactobacillus kitasatonis* (17.16%), and *Lactobacillus aviarius* (11.15%). In hens from CF system, *Lactobacillus kitasatonis* was the most abundant genus (34.29%), followed by *Romboutsia* (27.68%), and *Lactobacillus aviarius* (9.35%). The ten most abundant genera and their relative abundances by housing system can be found in Figure 3.

To determine specific OTU abundance differences across housing type, data were analyzed in SAS following abundance normalization which accounts for the number of sequence reads. Of the 200 OTUs analyzed, 64 OTUs were differentially abundant between housing types (Table 2 and 3). Eighteen OTUs were over-represented in CF compared to CC systems (Table 2). The majority of these OTUs were comprised of *Lactobacillus* sp. (5/18; 27.8%), *Staphylococcus* sp. (3/18; 16.7%), and *Corynebacterium* sp. (2/18; 11.1%) and did include over representation of OTUs that aligned to the *Lactobacillus kitasatonis* sequence at higher than 98% using BLAST (Altschul et al., 1990). This recently discovered bacterium has been isolated from the intestine, vagina, cloaca, and excreta of chickens (Mukai et al., 2003; Van Coillie et al., 2006; Yamazaki et al., 2012). While it has been studied for its ability to act as a probiotic and a competitive inhibitor of *Salmonella enteritidis* and *typhimurium*, it has not been shown to contribute significantly in either role (Van Coillie et al., 2006; Yamazaki et al., 2012).

The remaining 46 OTUs were over-represented in CC system (Table 3). The majority of these OTUs were comprised of *Romboutsia* sp. (9/46; 19.6%), *Lactobacillus* sp. (8/46; 17.4%),

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*Turicibacte*r sp. (7/46; 15%); *Peptostreptococcaceae* sp. (5/46; 10.9%) and *Clostridiales* sp. (5/46; 10.9%). As expected, many of the *Romboutsia* sp. were differently represented, with the closest BLAST aligned species being *Romboutsia timonensis* strain Marseille-P326. This strain was recently isolated in humans (Ricaboni et al., 2016). While it has been mentioned in poultry studies, it is largely unknown how this species is contributing to the chicken microbiota (Qiao et al., 2019, 2018). *Turicibacter sp.* have been identified with favorable feed conversion (low Residual Feed Intake) in both broiler male and females (Siegerstetter et al., 2017). Unfortunately, the current study did not explore hen production parameters such as hen day egg production or egg weight across the housing systems and cannot speculate on this relationship in hens. Among the species of *Clostridiales* identified, *Clostridioides difficile* was the only microorganism to be identified as a potential human pathogen. It composed an average of 1.08% of the abundance and a median of 0.00175%. This small percentage and even lower median indicate a few birds had high abundance while the majority had less than 0.002%.

Associations Between Intestinal Parameters and Microbial Communities

Correlation Summary. Overall, 48 correlations were identified for hens in CC systems and 43 correlations were identified for hens in CF systems with $r^2 > |0.35|$. For CC hens, 3 OTUs were associated with body weight (1 negative and 2 positive); 11 OTUs were associated with intestinal permeability (11 positive); 2 OTUs were associated with jejunal villus height (1 positive and 1 negative); 1 OTU was negatively associated with jejunal crypt depth; 3 were positively associated with jejunal villus height to crypt depth ratio; 16 OTUs were associated with ileal villus height (7 positive and 9 negative); 9 OTUs were associated with ileal crypt depth (1 positive and 8 negative); and 3 were positively associated with the ileal villus height to crypt depth ratio. For hens housed in CF systems, 1 OTU was negatively associated with body

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weight; 25 OTUs were associated with intestinal permeability (13 positive and 12 were negative); 2 OTUs were positively associated with jejunal crypt depth; 2 OTUs were negatively associated with ileal villus height; 9 OTUs were negatively associated with ileal crypt depth; and 3 were associated with the ileal villus height to crypt depth ratio (2 negative and 1 positive). Correlations with $r^2 > |0.35|$ can be found in Tables 4-7.

Correlation of OTUs with body weight. At the genus level, 1 *Lactobacillus* (*Lactobacillus aviarius*) OTU was negatively associated with body weight; while 2 *Romboutsia* OTUs (both showed highest similarity to *Romboutsia timonensis* strain Marseille-P326) were positively associated with body weight in the CC system. While no data has been reported regarding *Romboutsia timonensis* in chicken likely due to their recent identification (Ricaboni et al., 2016), ileal *Lactobacillus aviarius* has been associated with high feed conversion rates in broiler chicken (Stanley et al., 2012). While feed conversion rates in broilers are a relationship between feed to body weight gain, laying hens convert feed to egg production, which is energetically taxing. Therefore, maintaining certain body conditioning or body weight is imperative. The presence of *Lactobacillus aviarius* alone or in conjunction with *Romboutsia timonensis* may assist in this maintenance of body weight, but additional research is needed to understand this relationship. In CF, *Gallicola* sp. was the only microbe to be associated with body weight and was negatively associated with body weight. Unfortunately, our BLASTn search did not reveal a specific bacterium. Significant body weight correlations can be found in Table 4.

Correlation of OTUs with intestinal permeability. Intestinal permeability as measured by the rate of FITC-D flux from the intestine into circulation was positively associated with 4 *Lactobacillus*, 3 *Romboutsia*, 2 *Tyzzerella*, 1 *Turicibacter*, and 1 *Veillonellaceae* OTUs in hens

from CC systems and 6 *Lactobacillus*, and 1 *Megamonas*, *Gallicola*, *Corynebacterium*, *Staphylococcus*, *Dietzia*, and *Yaniella* OTUs in hens from CF systems. Significant intestinal permeability correlations can be found in Table 5.

While *Lactobacillus* sp. have been identified to have a protective nature in the intestine, the positive association between this genus and intestinal permeability would suggest that many species may have unfavorable impacts on intestinal health. Many of the positively associated species identified (*L. kitasatonis*, *L. mucosae*, *L. aviarius*, and *L. ingluviei* or *L. senmaizukei*) have been identified in poultry, but lack a described function (Qiao et al., 2019). The other genera have not been associated at the genus or species level to intestinal permeability. However, *Veillonellaceae* is a unique Firmicute in that it contains lipopolysaccharides (LPS) incorporated into its cell membrane (Marchandin and Jumas-Bilak, 2014) which have been recognized to stimulate the immune response, and increase intestinal permeability through decreasing tight junction proteins (Arce et al., 2010; Liu et al., 2012; Poltorak et al., 1998; Tanimura et al., 2008).

Interestingly, only negative correlations were identified in hens from CF systems, and included the following OTUs: 2 *Clostridiaceae*, 2 *Aeriscardovia*, 1 *Romboutsia*, 1 *Turicibacter*, and 1 *Gallibacterium*. Unlike with the positive correlations, two of these genera, *Gallibacterium anatis*, and two *Clostridium* (*C. nigeriense* and *C. chauvoei*), are associated directly with enteric disease or are potential pathogenic bacteria (Singh et al., 2016). Enteric disease in poultry has been associated with elevated intestinal permeability (Deng et al., 2012; Gilani et al., 2017a, 2017b; Vicuña et al., 2015). However, in this study, enteric disease was not identified, and our measure of intestinal permeability examines the whole intestinal tract and is not specific to the

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354 ileum where these microbial communities were isolated. Significant intestinal permeability
355 correlations can be found in Table 5.

356 ***Correlation of OTUs with jejunal intestinal morphology***. Jejunal morphology was
357 associated with a limited number of ileal bacterial communities across both housing systems. In
358 hens from CC systems, ileal microbial communities were associated with jejunal villus height (1
359 positive and 1 negative), jejunal crypt depth (1 negative; *Jeotgalicoccus* sp.) and jejunal villus
360 height to crypt depth ratio (3 positive). In hens from CF systems, 2 ileal microbial phylotypes
361 were positively associated with jejunal crypt depth (*Turicibacter sanguinis* and *Lactobacillus*
362 *acidophilus* or *Lactobacillus crispatus*). While *Lactobacillus acidophilus* has been used as a
363 probiotic (De Cesare et al., 2017; Forte et al., 2018), *Turicibacter sanguinis* is an
364 immunomodulating bacteria that may lead to secondary infections (Oh et al., 2017).
365 Additionally, *Turicibacter sanguinis* has been associated with bile salt reabsorption and intestinal
366 serotonin production; thus, it is unclear what role *Turicibacter sanguinis* has in regulating
367 intestinal physiology. Significant jejunal morphology correlations can be found in Table 6.

368 Among these limited correlations, ileal *Escherichia/Shigella* was negatively associated
369 with jejunal villus height in CC hens. This is not a surprising association as this taxonomic group
370 has been shown to cause detachment of villus tips; thus, reducing the overall size (Shi et al.,
371 2014). The other microbial phylotype associated with jejunal villus height, *Tyzzerella* sp., was
372 positively associated with jejunal villus height and jejunal villus height to crypt depth ratio. In
373 poultry, *Tyzerella* sp. abundance was elevated with probiotic supplementation (Gao et al., 2017),
374 which has been associated with increasing villus height (Heak et al., 2017).

375 The two remaining species for jejunal villus height to crypt depth ratio were
376 *Streptococcus* sp. and *Lactobacillus* sp. Interestingly, both genera have been used to formulate

probiotics (De Cesare et al., 2017; Forte et al., 2018; Hanchi et al., 2018; Mallo et al., 2010) and the specific *Lactobacillus* sp. (*Lactobacillus acidophilus* and *Lactobacillus crispatus)* has been associated with increased jejunal villus height when administered in the feed (Chae et al., 2012; De Cesare et al., 2017; Forte et al., 2018). While this study identified correlations between ileal microbial communities and jejunal morphology, the number of correlations were limited suggesting that local or site-specific microbial communities likely play a larger role in shaping the intestinal physiology than presence in other areas of the intestinal tract. Additionally, this highlights the importance of characterizing site-specific communities and cautiously assigning interpretations across intestinal sections.

Correlation of OTUs with ileal intestinal morphology. As expected, the greatest number of correlations for ileal OTUs were found with ileal intestinal morphology. See Table 7 for correlations between ileal microbial communities and ileal intestinal morphology with $r^2 > |0.35|$. In hens from CC systems, 16 OTUs associated with ileal villus height, 9 were negatively correlated and 7 were positively correlated; and 2 OTUs were negatively associated with ileal villus height in hens from CF systems. The majority of the OTUs associated with ileal villus height were: *Lactobacillus* (4 negative, 5 positive; 8 CC and 1 CF). The negatively correlated *Lactobacillus sp.* included L. *acidophilus*, *L. aviarius*, and L. *collinoides.* A single OTU, OTU 175, which aligned to *L. aviarius*, was negatively correlated across both housing types. Unfortunately, much remains unknown regarding the function of *L. aviarius*. The remaining negatively correlated OTUs were *Enterococcus, Campylobacter, Fusobacterium, Gallibacterium (G. anatis),* and *Clostridium (C. cuniculi or C. saudinese*). *Campylobacter* and *Gallibacterium anatis* have been associated with either primary or secondary enteric diseases (Singh et al., 2016). Additionally, several *Enterococcus sp.* and *Clostridium sp.* have been associated with

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enteric diseases, but the particular phylotypes identified here have not been associated with enteric disease. The positively correlated OTUs included the genera *Atopobium* and *Bifidobacterium* and of the positively associated OTUs, the *Lactobacillus* species included L. *acidophilus*, *L. aviarius*, and *L. kitasatonis*. As previously mentioned, *L. acidophilus* is the only of the *Lactobacillus* species that have been associated with improved intestinal health (Brisbin et al., 2011). These correlations agree with published functional data suggesting that these analyses are correctly identifying known bacterial genera and species with local morphometric changes.

Ileal crypt depth was associated with 9 OTUs for each of the two hen housing types. Interestingly in the CC system, 1 of the 9 OTUs was positively correlated with crypt depth; whereas none of the 9 OTUs were positively correlated with crypt depth in the CF system. The only positively correlated OTU was identified as a group of bacteria known as *Candidatus Arthromitus* or segmented filamentous bacteria (SFB). This group of bacteria are known to positively stimulate the gastrointestinal immune system (Bolotin et al., 2014), which could be through the expansion of the crypt (Flannigan and Denning, 2018; Schnupf et al., 2017). The eight negatively associated OTUs were associated with 8 different genera in hens from CC. OTUs of interest are known pathogenic bacteria such as *Campylobacter jejuni*, *Helicobacter winghaensi*, and potential pathogenic bacteria, *Clostridium difficile*, and *Lactobacillus avarius*. In the CF system, the 9 negatively associated OTUs were grouped into 3 genera: *Romboutsia* (4 OTUs); *Lactobacillus.* (3); and *Clostridium* (2). Of the *Lactobacillus* OTUs only 1 is a previously discussed in this manuscript, *L. aviarius*, and lacks previous research to agree or disagree with our finding. The remaining *Lactobacillus* phylotypes consist of *L. taiwanesis* or *L. gasseri*, or *L. johnsonii*, *L. delbrueckii bulgaricus*. *L. taiwanesis* or *L. gasseri*, or *L. johnsonii* have been identified in bobwhite quail and may serve a role in fatty acid and carbohydrate

metabolism (Zhang et al., 2017) and *L. delbrueckii bulgaricus* has been associated with cheese and yogurt production (El Kafsi et al., 2014). While our correlations indicate the presence of these bacteria may decrease crypt depth, this may be a result of change in ileal digestibility that reduces the overall need to proliferate in the crypts.

A limited number of OTUs were correlated with villus height to crypt depth ratio. Each system had 3 different OTUs associated with villus height to crypt depth ratio. In the CC system, 3 OTUs, all from *Lactobacillus*, were positively associated with ileal villus height to crypt depth ratio. Two of the OTUs were associated with *L. kitasatonis* and the other has high similarity to *L. aviarius*; both of which have limited functional data in previously published literature. In the CF system, 2 of the 3 OTUs correlated with ileal villus height to crypt depth ratio were negatively associated. These species included *L. aviarius* and *Clostridium nigeriense* and based on limited research it is unclear if either of the bacteria have been associated with intestinal health markers (Alou et al., 2017). *L. mucosae* was positively associated with ileal villus height to crypt depth ratio. Again, limited information is available regarding *L. mucosae* however, this favorable correlation may provide some insight into the functionality.

The increased number of correlations between ileal OTUs and ileal morphology compared to jejunal morphology would indicate the importance of using site specific microbial community data to assess influential communities. While this study identified several different phylotypes that were associated with ileal morphology, causation cannot be assessed from this study. Additional studies are needed to confirm these associations across these housing systems and to define the roles these phylotypes have in the intestinal physiology and overall performance of the hen.

Conclusions

This study investigated changes of intestinal health of commercial laying hens under optimal commercial conditions for each system. This is the first study to determine intestinal physiology, ileal communities and association between intestinal physiology and ileal communities in hens across different commercial layer housing systems. In this study, we have identified greater changes in intestinal morphology in the jejunum compared to ileum. However, favorable villus height to crypt depth ratios in both the jejunum and ileum were observed in hens from CC systems, suggesting a balance in the production and sloughing of the intestinal epithelial lining in the CC hens. However, it should be noted in both groups, ranges for villus height, crypt depth and their ratio were similar to previous reports and these changes are likely only contributing to more efficient production. Additionally, the measurement of the macromolecule, FITC-D, was lowly detected and similar across housing types suggesting minimal intestinal permeability. Ileal bacterial community diversity measurements were different and favored hens housed in the CC types due to increased species richness, alpha diversity, and over- represented OTUs. Despite the increased over-represented OTUs in CC systems, neither housing type had a significant number of over-represented known pathogenic bacteria. Lastly, we explored the correlation of bacterial communities with intestinal traits. A primary finding of this study was that a higher number of correlations were observed between ileal morphology and ileal microbial communities compared to jejunal morphology and ileal microbial communities. This suggests the site-specific microbial community contributes to the intestinal environment and comparisons across even segments of the small intestine should be limited. Additionally, this study identified several OTUs that were associated with these traits as expected; thus, providing validation for correlations where OTUs have limited information. For example, *L. acidophilus* phylotypes positively correlated with ileal villus height or an OTU associated with

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469 *Escherichia/Shigella* negatively correlated with jejunal villus height. In conclusion, these results
470 were obtained from a commercial setting instead of a controlled research environment where one
471 system is generally disadvantaged. Several parameters were found to be more favorable for hens
472 housed in CC suggesting an advantage of this system for intestinal health of these hens.
473 However, it should be pointed out that the lower intestinal health parameters observed in CF
474 were not at levels to indicate detrimental effects (e.g. similar macromolecular flux and
475 pathogenic bacteria), but the differences may highlight known reduced efficiencies of the CF
476 system (e.g. villus height to crypt depth ratio; microbial diversity). However, additional studies
477 are needed to characterize these potentially beneficial bacterial interactions with the hen intestine
478 across housing types to determine if these relationships can be obtained for both systems.
479

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Table 1. Least squared means for intestinal parameters from hens housed in conventional cage and cage-free housing systems.

Section	Parameter	Unit	CC	CF	*SEM*	P-value
Whole Intestine	Permeability	ng/ml	114.23	101.31	*9.8*	0.348
		Fluor.	971.69	866.68	*199.16*	0.709
Jejunum	Villus Height	µM	755.21	866.08	*25.05*	0.002
	Crypt Depth	µM	121.52	156.34	*1.96*	<0.001
	Ratio	µM/µM	6.60	5.94	*0.25*	0.064
Ileum	Villus Height	µM	591.89	572.94	*9.90*	0.175
	Crypt Depth	µM	117.62	127.85	*5.03*	0.151
	Ratio	µM/µM	5.30	4.84	*0.19*	0.091
	Goblet number	Count/mm^{2}[a]	1170	686	*74*	<0.001

Abbreviations: CC, conventional cage housing system; CF, cage-free housing system; SEM, Standard error of the means, Fluor., Fluorescence

[a] Goblet cell count per area of each image.

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673 Table 2. Operational Taxonomic Units overrepresented in ileal digesta of hen housed in a commercial cage free system.

Group[a]	Fold Change[b]	P-value	q-value	Taxonomy[c]	Taxonomy based on NCBI BLASTn Search[d]
Otu00036	1.5374	0.0054	0.0183	*Streptococcus*	*Strep. alactolyticus; S. griseocameus; S. gallolyticus; S. macedonicus; S. pateurianus*
Otu00004	1.5394	0.0169	0.0382	*Tyzzerella*_3	-
Otu00168	1.5625	0.0063	0.0200	*Lactobacillus*	*Lactobacillus kitasatonis*
Otu00191	1.6654	0.0127	0.0310	*Nocardiopsis*	*Nocardiopsis alkaliphila; N. kunsanensis*
Otu00110	1.7306	0.0041	0.0150	*Staphylococcus*	*Staphylococcus lentus; S. sciuri*
Otu00055	1.7452	0.0002	0.0022	*Staphylococcus*	*Staphylococcus equorum*
Otu00135	1.7470	0.0005	0.0032	*Lactobacillus*	*Lactobacillus acidophilus; L. crispatus*
Otu00161	1.7958	0.0182	0.0400	*Bacteroides*	*bacteroides salanitronis*
Otu00116	1.8070	0.0007	0.0045	*Lactobacillus*	*Lactobacillus secaliphilus*
Otu00157	1.8490	0.0074	0.0221	*Yaniella*	*Yaniella halotolerans*
Otu00087	1.8546	0.0009	0.0048	*Staphylococcaceae*_unclassified	*Salinicoccus kekensis; S. gingdaonensis; S. alkaliphilus*
Otu00166	1.9298	0.0046	0.0160	*Helicobacter*	*Helicobacter winghamenss; H. pametensi; H. macacae; H. brantae*
Otu00096	2.0089	0.0003	0.0022	*Dietzia*	*Dietzia lutea; D. timorensis*
Otu00072	2.0570	0.0008	0.0048	*Lactobacillus*	*Lactobacillus hayakitensis*
Otu00035	2.4141	0.0074	0.0221	*Aeriscardovia*	-
Otu00054	2.4993	0.0000	0.0001	*Corynebacterium*_1	*Corynebacterium singular; C. sphenisorum; C. glyciniphilum; C. minutissimum*
Otu00050	2.8528	0.0003	0.0024	*Corynebacterium*_1	*Corynebacterium casei; C. ammoniagenes*

674 [a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 200
675 taxonomic groups.
676 [b] Fold change is expressed relative to CC system.
677 [c] Taxonomic assignments are based the SILVA SSU NR reference database (v 132).
678 [d] BLASTn search results were reported if the similarity was higher than 97%. – indicates sequence alignments of less than 97%.
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680 Table 3. Operational Taxonomic Units overrepresented in ileal digesta of hen housed in a commercial conventional cage system.

Group[a]	Fold Change[b]	P-value	q-value	Taxonomy[c]	Taxonomy based on NCBI BLASTn Search[d]
Otu00062	0.1670	0.0001	0.0012	*Clostridiaceae*_1_unclassified	*Clostridium fallax* strain DSM 2631*; C. chauvoei* strain DSM 7528
Otu00134	0.2049	<0.0001	0.0001	*Peptostreptococcaceae*_unclassified	*Romboutsia timonensis* strain Marseille-P326
Otu00127	0.2130	0.0003	0.0022	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Otu00142	0.2242	0.0001	0.0016	*Turicibacter*	*Turicibacter sanguinis*
Otu00148	0.2291	0.0010	0.0048	*Peptostreptococcaceae*_unclassified	*Clostridioides difficile*
Otu00152	0.2418	<0.0001	0.0004	*Peptostreptococcaceae*_unclassified	*Terrisporobacter othiniensis; Peptostreptococcaceae bacterium*
Otu00197	0.2513	0.0004	0.0029	*Peptostreptococcaceae*_unclassified	*Clostridioides difficile*
Otu00115	0.2622	0.0017	0.0075	*Tyzzerella*_3	*Turicibacter sanguinis*
Otu00080	0.2750	0.0003	0.0022	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Otu00108	0.2872	0.0002	0.0022	*Turicibacter*	*Turicibacter sanguinis*
Otu00067	0.2906	0.0005	0.0034	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Otu00079	0.2939	0.0001	0.0017	*Clostridiales*_unclassified	*Corynebacterium atypicum; C. pseudogenitalium*
Otu00154	0.2941	0.0017	0.0075	*Clostridiaceae*_1_unclassified	*Clostridium nigeriense* strain Marseille-P2414T
Otu00084	0.3151	0.0011	0.0053	*Turicibacter*	*Turicibacter sanguinis*
Otu00170	0.3190	<0.0001	<0.0001	*Corynebacterium*_1	*Corynebacterium glutamicum; C. efficiens*
Otu00085	0.3260	0.0009	0.0048	*Turicibacter*	*Turicibacter sanguinis*
Otu00189	0.3385	0.0001	0.0012	*Turicibacter*	*Turicibacter sanguinis*
Otu00109	0.3819	0.0075	0.0221	*Bacteroides*	-
Otu00178	0.3833	0.0110	0.0275	*Bacteroides*	-
Otu00019	0.3919	0.0019	0.0081	*Peptostreptococcaceae*_unclassified	*Clostridioides difficile*
Otu00039	0.4178	0.0038	0.0142	*Clostridiaceae*_1_unclassified	*Clostridium chauvoei; C. tertium; C. sartagoforme*
Otu00183	0.4382	0.0171	0.0382	*Turicibacter*	*Turicibacter sanguinis*
Otu00029	0.4463	<0.0001	0.0008	*Terrisporobacter*	*Terrisporobacter othiniensis*

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Otu00100	0.4651	0.0002	0.0022	*Gallicola*	*uncultured bacterium*
Otu00123	0.4713	0.0033	0.0133	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Otu00031	0.4997	<0.0001	0.0007	*Lactobacillus*	*Lactobacillus ingluviei*
Otu00010	0.5207	0.0012	0.0057	*Turicibacter*	*Turicibacter sanguinis*
Otu00091	0.5290	0.0088	0.0240	*Lactobacillus*	*Lactobacillus aviarius*
Otu00200	0.5319	0.0220	0.0474	*Ruminococcaceae*_UCG-005	-
Otu00175	0.5350	0.0009	0.0048	*Lactobacillus*	*Lactobacillus aviarius*
Otu00113	0.5452	0.0042	0.0150	*Aeriscardovia*	*Aeriscardovia aeriphila*
Otu00009	0.5533	0.0095	0.0254	*Lactobacillus*	*Lactobacillus acidophilus; L. crispatus*
Otu00195	0.5830	0.0105	0.0269	Candidatus_*Arthromitus*	-
Otu00198	0.5884	0.0079	0.0229	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Otu00077	0.5951	0.0170	0.0382	*Lactobacillus*	*Lactobacillus ingluviei; L. senmaizukei*
Otu00151	0.6035	0.0149	0.0351	*Lactobacillales*_unclassified	*Lactobacillus pobuzihii*
Otu00027	0.6168	<0.0001	<0.0001	*Aeriscardovia*	*Aeriscardovia aeriphila*
Otu00086	0.6299	0.0037	0.0142	*Lactobacillus*	*Lactobacillus agilis*
Otu00094	0.6319	0.0063	0.0200	*Clostridiales*_unclassified	*Romboutsia timonensis* strain Marseille-P326
Otu00137	0.6434	0.0111	0.0275	*Lactobacillus*	*Lactobacillus mucosae*
Otu00185	0.6456	0.0059	0.0197	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Otu00068	0.6604	0.0228	0.0486	Candidatus_*Arthromitus*	-
Otu00130	0.6655	0.0082	0.0232	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Otu00114	0.6842	0.0083	0.0232	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Otu00025	0.7166	0.0099	0.0260	*Romboutsia*	*Romboutsia weinsteinii*
Otu00028	0.7943	0.0147	0.0351	*Lactobacillus*	*Lactobacillus agilis*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 200 taxonomic groups.

[b] Fold change is expressed relative to CC system.

[c] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 132).

[d] BLASTn search results were reported if the similarity was higher than 97%. – indicates sequence alignments of less than 97%.

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686 Table 4. Correlation of Operational Taxonomic Units from ileal digesta in hen from conventional cage and cage free systems for body weight.
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Housing	Group[a]	Correlation	Taxonomy[b]	Taxonomy based on NCBI BLASTn Search[c]
Conventional Cage	Otu00128	-0.35	*Lactobacillus*	*Lactobacillus aviarius*
	Otu00136	0.38	*Peptostreptococcaceae*	*Romboutsia timonensis* strain Marseille-P326
	Otu00198	0.47	*Romboutsia*	*Romboutsia timonensis strain Marseille-P326*
Cage-free	Otu00100	-0.39	*Gallicola*	*uncultured bacterium*

690 [a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 200
691 taxonomic groups.
692 [b] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 132).
693 [c] BLASTn search results were reported if the similarity was higher than 97%. – indicates sequence alignments of less than 97%.
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695
696 Table 5. Correlation of Operational Taxonomic Units from ileal digesta in hen from conventional cage and cage-free systems for intestinal
697 permeability[a].

Housing	Group[b]	Correlation	Taxonomy[c]	Taxonomy based on NCBI BLASTn Search[d]
Conventional Cage	Otu00126	0.36	*Lactobacillus*	*Lactobacillus kitasatonis*
	Otu00137	0.39	*Lactobacillus*	*Lactobacillus mucosae*
	Otu00032	0.39	*Veillonellaceae*	*Veillonella magna*
	Otu00082	0.40	*Turicibacter*	*Turicibacter sanguinis*
	Otu00144	0.44	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
	Otu00193	0.49	*Peptostreptococcaceae*	*Romboutsia timonensis* strain Marseille-P326
	Otu00174	0.50	*Lactobacillus*	*Lactobacillus aviarius*
	Otu00077	0.50	*Lactobacillus*	*Lactobacillus ingluviei; senmaizukei*
	Otu00064	0.53	*Tyzzerella*_3	-
	Otu00004	0.57	*Tyzzerella*_3	*Natranaerovirga pectinivora* strain DSM 24629
	Otu00119	0.59	*Romboutsia*	*Romboutsia timonensis* strain Marseille-P326
Cage-free	Otu00057	0.35	*Megamonas*	*Megamonas funiformis*
	Otu00100	0.36	*Gallicola*	*uncultured bacterium*
	Otu00054	0.36	*Corynebacterium*_1	*Corynebacterium singular; C. sphenisorum; C. glyciniphilum; C. minutissimum*
	Otu00055	0.37	*Staphylococcus*	*Staphylococcus equorum*
	Otu00096	0.40	*Dietzia*	*Dietzia lutea*
	Otu00120	0.41	*Lactobacillus*	*Lactobacillus* bacterium isolate MGYG-HGUT-01336
	Otu00157	0.44	*Yaniella*	*Yaniela halotolerans*
	Otu00011	0.44	*Lactobacillus*	*Lactobacillus vaginalis*
	Otu00059	0.53	*Lactobacillus*	*Lactobacillus mucosae*
	Otu00186	0.53	*Peptococcus*	--
	Otu00101	0.59	*Lactobacillus*	*Lactobacillus oris; L. panis; L. antri; Lfrumenti; L. reuteri*
	Otu00081	0.62	*Lactobacillus*	*Lactobacillus kitasatonis*

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Otu00023	0.67	*Lactobacillus*	*Lactobacillus mucosae*
Otu00117	-0.50	*Aeriscardovia*	
Otu00187	-0.45	*Lactobacillus*	*Lactobacillus kitasatonis; L. pasteurii*
Otu00035	-0.41	*Aeriscardovia*	--
Otu00154	-0.41	*Clostridiaceae*_1_unclassified	*Clostridium nigeriense*
Otu00134	-0.41	*Peptostreptococcaceae*_unclassified	*Romboutsia timonensis*
Otu00153	-0.40	*Gallibacterium*	*Gallibacterium anatis*
Otu00039	-0.40	*Clostridiaceae*_1_unclassified	*Clostridium chauvoei*
Otu00147	-0.39	*Lactobacillus*	*Lactobacillus aviarius*
Otu00158	-0.38	*Romboutsia*	*Romboutsia timonensis*
Otu00175	-0.38	*Lactobacillus*	*Lactobacillus aviarius*
Otu00021	-0.38	*Lactobacillus*	*Lactobacillus collinoides; L. siliginis; L. paracollinoides*
Otu00082	-0.35	*Turicibacter*	*Turicibacter sanguinis*

[a] Permeability measured in fluorescence.
[b] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 200 taxonomic groups.
[c] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 132).
[d] BLASTn search results were reported if the similarity was higher than 97%. – indicates sequence alignments of less than 97%.

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Table 6. Correlation of Operational Taxonomic Units from ileal digesta in hen from conventional cage and cage-free systems for jejunal morphology.

Intestinal parameter	Housing	Group[a]	Correlation	Taxonomy[b]	BLAST Search[c]
Villus height	Conventional Cage	Otu00046	-0.40	*Escherichia-Shigella*	*Clostridium cuniculli; Blastococcus litoris; E. coli O157H7; Escherichia albertii KF1; Shigella boydii*
		Otu00058	0.40	*Tyzzerella*_3	-
	Cage-free	-	-	-	-
Crypt Depth	Conventional Cage	Otu00190	-0.35	*Jeotgalicoccus*	*Jeotgalicoccus halotoleran; J. halophilus; J. saudimassiliensis*
	Cage-free	Otu00143	0.36	*Lactobacillus*	*L. acidophilus; L. crispatus*
		Otu00082	0.43	*Turicibacter*	*Turicibacter sanguinis*
Villus Height to Crypt Depth Ratio	Conventional Cage	Otu00173	0.36	*Streptococcus*	*Streptococcus hyovafinalis; S. acidominimus*
		Otu00088	0.47	*Lactobacillus*	*Lactobacillus acidophilus; L.s crispatus*
		Otu00058	0.48	*Tyzzerella*_3	-
	Cage-free	-	-	-	-

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 200 taxonomic groups.
[b] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 132).
[c] BLASTn search results were reported if the similarity was higher than 97%. – indicates sequence alignments of less than 97%.

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710 Table 7. Correlation of Operational Taxonomic Units from ileal digesta in hen from CC and CF systems for ileal morphology.
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Intestinal parameter	Housing	Group[a]	Correlation	Taxonomy[b]	Taxonomy based on NCBI BLASTn Search[c]
Villus Height	Conventional Cage	Otu00043	-0.41	*Peptostreptococcaceae*	*Romboutsia timonensis* strain Marseille-P326
		Otu00111	-0.41	*Enterococcus*	*Enterococcus lactis; villorum, canis, cinnamoneus, canintestini; faecium*
		Otu00041	-0.41	*Campylobacter*	*Campylobacter insulaenigrae, C. armoricus, C. helveticus*
		Otu00013	-0.38	*Fusobacterium*	*Fusobacterium necrogenes, mortiferum, mvarium, ulcerans*
		Otu00008	-0.38	*Gallibacterium*	*Gallibacterium anatis*
		Otu00175	-0.37	*Lactobacillus*	*Lactobacillus aviarius*
		Otu00021	-0.36	*Lactobacillus*	*Lactobacillus collinoides; L. siliginis; L. paracollinoides*
		Otu00016	-0.36	*Clostridium*_sensu_stricto_1	*Clostridium cuniculi; saudiense*
		Otu00150	-0.35	*Lactobacillus*	*Lactobacillus aviarius; L. acidioiscis*
		Otu00131	0.36	*Lactobacillus*	*Lactobacillus kitasatonis*
		Otu00009	0.38	*Lactobacillus*	*Lactobacillus acidophilus; Lactobacillus crispatus*
		Otu00091	0.39	*Lactobacillus*	*Lactobacillus aviarius*
		Otu00122	0.40	*Atopobium*	-
		Otu00018	0.41	*Lactobacillus*	*Lactobacillus gigerionum; amylolytics*
		Otu00118	0.42	*Bifidobacteriaceae*	*Bifidobacterium commune*
		Otu00156	0.46	*Lactobacillus*	*Lactobacillus kitasatonis; L. acidophilus*
	Cage-free	Otu00141	-0.41	*Romboutsia*	*Romboutsia timonensis*
		Otu00175	-0.35	*Lactobacillus*	*L. aviarius*
Crypt Depth	Conventional Cage	Otu00110	-0.40	*Staphylococcus*	*Staphylococcus lentus; sciuri*
		Otu00150	-0.38	*Lactobacillus*	*Lactobacillus aviarius; L. acidioiscis*
		Otu00166	-0.38	*Helicobacter*	*Helicobacter winghamenss; pametensis, macacae, brantae*
		Otu00106	-0.37	*Campylobacter*	*Campylobacter upsaliensis; coli, jejuni sp*

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		Otu00146	-0.36	*Phascolarctobacterium*	*Chryseobacterium oncorhynchi*
		Otu00179	-0.36	*Parasutterella*	-
		Otu00157	-0.36	*Yaniella*	*Yaniella halotolerans*
		Otu00148	-0.35	*Peptostreptococcaceae*	*Clostridioides difficile*
		Otu00180	0.43	Candidatus_*Arthromitus*	-
	Cage-free	Otu00141	-0.48	*Romboutsia*	*Romboutsia timonensis*
		Otu00048	-0.41	*Clostridium*_sensu_stricto_1	*Clostridium disporicum*
		Otu00012	-0.41	*Lactobacillus*	*L. taiwanensis; L.hominis; L. Paragasseri; L. gasseri; L. johnsonii*
		Otu00047	-0.41	*Lactobacillus*	*Lactobacillus* isolate MGYG-HGUT-01336
		Otu00069	-0.41	*Romboutsia*	*Romboutsia timonensis*
		Otu00016	-0.39	*Clostridium*_sensu_stricto_1	*Clostridium cuniculi; saudiense*
		Otu00025	-0.38	*Romboutsia*	*Romboutsia weinsteinii*
		Otu00038	-0.36	*Romboutsia*	*Romboutsia timonensis*
		Otu00014	-0.35	*Lactobacillus*	*L. delbrueckii bulgaricus*
Villus Height to Crypt Depth Ratio	Conventional Cage	Otu00168	0.35	*Lactobacillus*	*Lactobacillus kitasatonis*
		Otu00176	0.39	*Lactobacillus*	*Lactobacillus aviarius*
		Otu00156	0.39	*Lactobacillus*	*Lactobacillus aviarius; L. acidioiscis*
	Cage-free	Otu00163	-0.43	*Lactobacillus*	*Lactobacillus aviarius*
		Otu00154	-0.37	*Clostridiaceae*_1_unclassified	*Clostridium nigeriense*
		Otu00059	0.35	*Lactobacillus*	*Lactobacillus mucosae*

[a] Group denotes the taxonomic group assigned to each unique sequence. This table only includes those significantly different from the top 200 taxonomic groups.

[b] Taxonomic assignments are based on sequence similarity to the SILVA SSU NR reference database (v 132).

[c]BLASTn search results were reported if the similarity was higher than 97%. – indicates sequence alignments of less than 97%.

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Figure 1. *Boxplots of alpha diversity measurements of ileal microbiota from hens in commercial conventional cage (CC) and cage free (CF) systems*. Goldenrod denotes the diversities from hens in CF systems and red denotes the diversities from hens in CC.

Figure 2. *Principle Coordinate Analysis (PCoA) comparing the ileal microbiota of hens in commercial conventional cage (CC) and cage free (CF) systems*. Goldenrod denotes hens in CF systems and red denotes hens in CC systems.

Figure 3. *The 10 most abundant ileal microbiota operational taxonomic units (OTUs) by commercial housing system.* Percentages of the top 10 OTUs are represented based on abundances for each commercial housing system. Each OTU genera or species identification can be found in the figure legend.

Supplemental Figure 1. Distribution of phyla by housing type.

Supplemental Figure 2. Distribution of genera by housing type.

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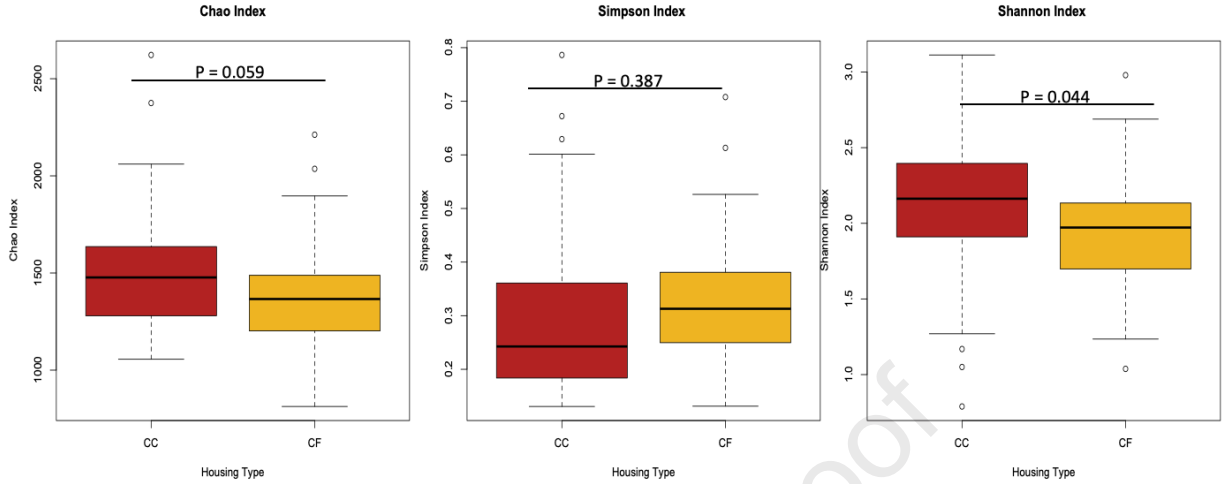

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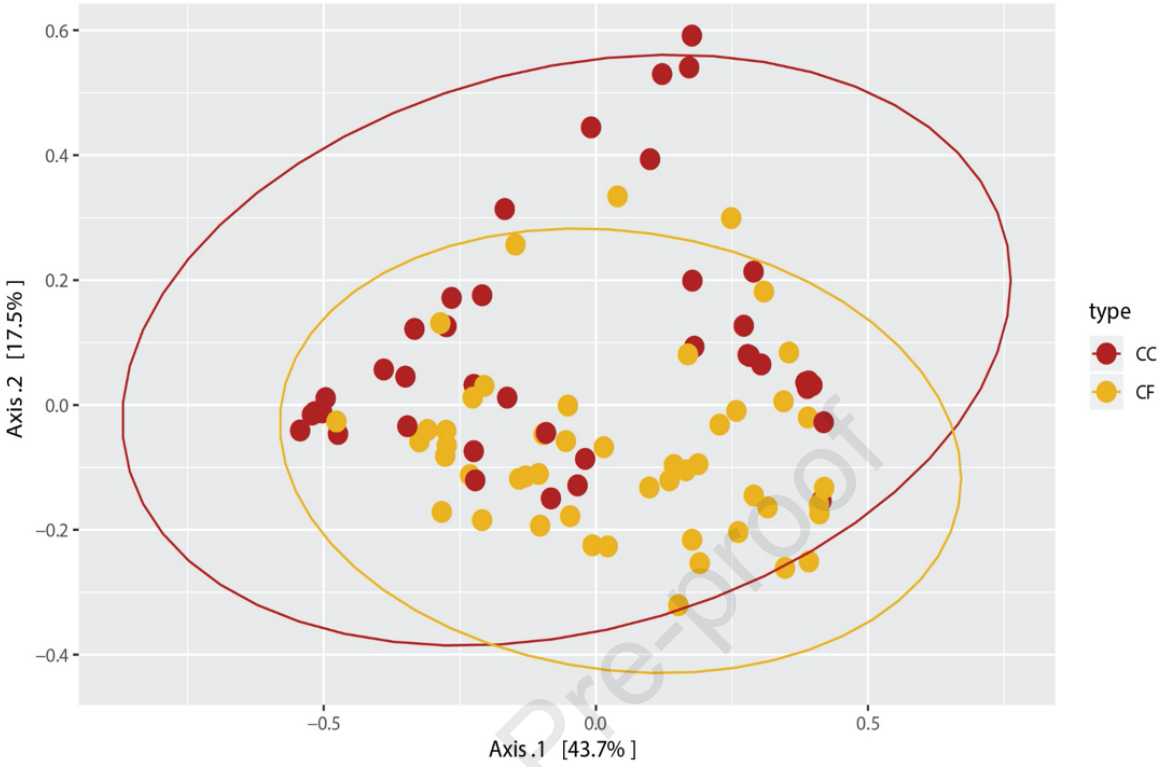

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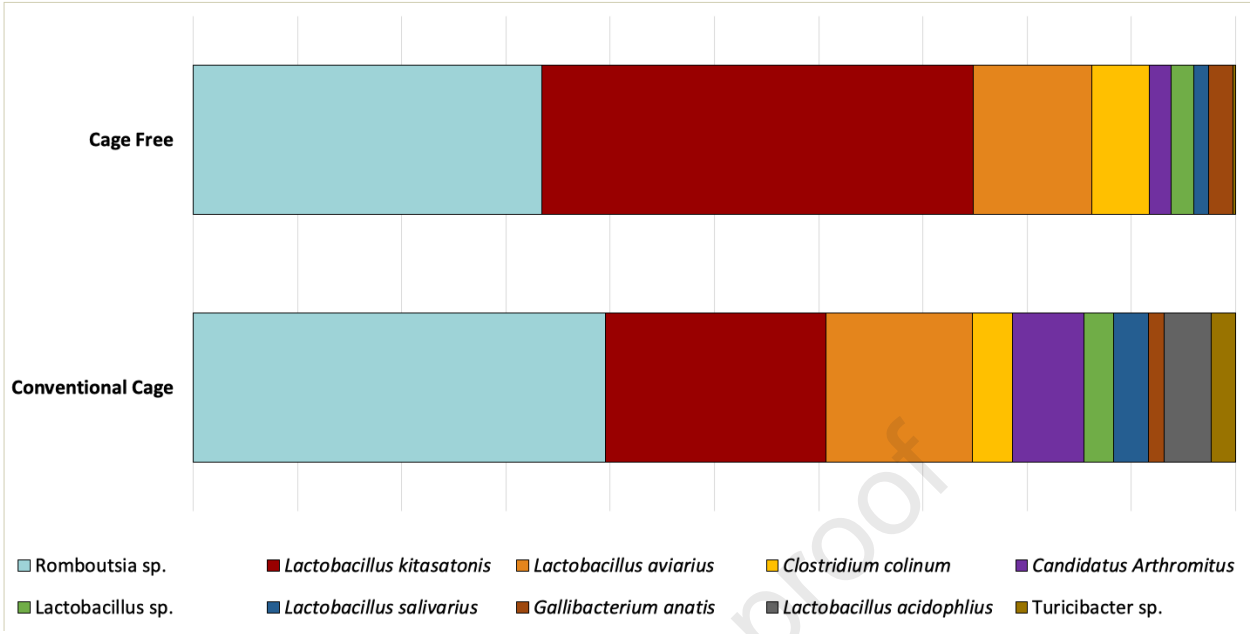

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The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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